

Effects of Sildenafil on Oxidative and Inflammatory Injuries of the Kidney in Streptozotocin-Induced Diabetic Rats

Kyung-Hwan Jeong^a Tae-Won Lee^a Chun-Gyoo Ihm^a Sang-Ho Lee^a
Ju-Young Moon^a Sung-Jig Lim^b

Departments of ^aNephrology and ^bPathology, School of Medicine, Kyung Hee University, Seoul, Korea

Key Words

Diabetic nephropathy · Inflammation · Oxidative stress · Sildenafil

Abstract

Background: Oxidative stress and inflammation are implicated in the pathogenesis of diabetic nephropathy. Because sildenafil citrate (Viagra[®]) has variable cardiovascular benefits, including antioxidative and immunomodulating effects, we investigated its influence on oxidative stress and inflammation in diabetic rat kidney. **Methods:** Streptozotocin-induced diabetic rats received sildenafil (3 mg/kg/day in drinking water) or not (undosed water) for 8 weeks and were compared to age-matched nondiabetic animals. We evaluated 8-hydroxydeoxyguanosine (8-OHdG; for oxidative DNA damage), inducible nitric oxide synthase (iNOS) and nitrotyrosine (for excessive NO production and peroxynitrite formation), and representative chemoattractants [monocyte chemoattractant protein-1, MCP-1; for inflammation and monocyte/macrophage infiltrations (ED-1)] in the kidney. **Results:** Sildenafil-treated rats had a lower kidney-to-body weight ratio than untreated diabetic rats. Urinary albumin excretion in diabetic rats decreased significantly after sildenafil treatment without changes in systolic blood pressure. Sildenafil-

treated rats had significantly lower urinary and renal cortical 8-OHdG levels than the nonsildenafil group. Sildenafil administration significantly attenuated the increased renal nitrotyrosine protein expression, positive iNOS and ED-1 staining in glomeruli and tubulointerstitium, and nitrotyrosine staining in tubulointerstitium. Cortical MCP-1 RNA expression in the sildenafil group was significantly lower than in the nonsildenafil group. **Conclusions:** Sildenafil treatment may attenuate renal damage by ameliorating oxidative and inflammatory injuries in diabetic rats.

Copyright © 2008 S. Karger AG, Basel

Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide. Diabetes has been linked to enhanced reactive oxygen species (ROS) production [1]. Increased ROS can cause vascular endothelium abnormalities, reacting directly with nitric oxide (NO) to produce cytotoxic peroxynitrite and thus increasing reactivity to vasoconstrictors and modification of extracellular matrix proteins [2, 3]. ROS can also damage endothelial cells indirectly by stimulating expression of various genes involved in inflammatory pathways [4].

Macrophages that migrate into renal tissue could cause structural damage through the release of proinflammatory and profibrotic cytokines as well as production of ROS [5, 6]. Endothelial dysfunction is also thought to play a pivotal role in the development of DN and related oxidative stress via upregulating ROS formation [7]. Therefore, DN is thought to result from interactions between these metabolic and hemodynamic factors [5, 7, 8].

Sildenafil (Viagra®; Pfizer, New York, N.Y., USA), a type-5 phosphodiesterase (PDE-5) inhibitor which increases cGMP levels in response to NO, augments the relaxation of vascular smooth muscle [9]. This drug has been used to treat erectile dysfunction [10]; however, novel therapeutic indications are emerging with the discovery that PDE-5 is expressed in various tissues, such as vascular and bronchial smooth muscles and platelets [11, 12]. Furthermore, there is abundant PDE-5 expression and activity in the kidney, which suggests that selective inhibition of PDE-5 may be advantageous in treating variable kidney disease [13]. In recent years, a beneficial effect of sildenafil has been reported for immediate post-transplantational warm ischemic kidney injury [14]. Rodriguez-Iturbe et al. [15] also reported that sildenafil treatment prevented hypertension and deterioration of renal function, reduced inflammation and delayed the onset of proteinuria in rats with 5/6 nephrectomy. Much evidence suggests the protective effects of sildenafil against diverse injuries in the kidney and endothelial system, but studies demonstrating an association between sildenafil treatment and amelioration of diabetic renal injury are rare.

Moreover, sildenafil has been shown to reduce oxidative stress via inhibition of superoxide formation in vitro and to decrease the inflammatory response via improving oxygenation in vivo [15, 16]. Although other reports have shown the protective effect of sildenafil in ischemic kidney injury models [14, 15], the renoprotective potential of this drug against oxidative stress and inflammation in streptozotocin (STZ)-induced diabetic rats has not been reported to date.

We hypothesized that sildenafil administration in STZ-induced diabetic rats may exert direct or indirect effects via improving oxidative and inflammatory injury beyond PDE-5 inhibition and thus ameliorate DN. 8-Hydroxydeoxyguanosine (8-OHdG) is a product of oxidative DNA damage following specific enzymatic cleavage after ROS-induced 8-hydroxylation of the guanine base in mitochondria and nuclear DNA [17]. Peroxynitrite oxidizes DNA and can inactivate proteins by forming 3-nitrotyrosine residues [18]. Moreover, 8-OHdG and nitro-

tyrosine are directly or indirectly involved in enhanced superoxide generation and oxidative damage in the kidney in diabetes [19, 20]. Inducible NO synthase (iNOS), an enzyme expressed in all nucleated cells, generates large bursts of NO in response to various pathologic stimuli [21, 22]. Because iNOS is responsible for the production of sustained high levels of NO, it is often considered the primary culprit of autotoxicity under oxidative stress. Indeed, greater expression of iNOS has been found in cardiovascular disease, coupled to alterations in NO availability and inflammatory processes [23]. Chemoattractants, such as monocyte chemoattractant protein (MCP-1), mainly mediate the influx of inflammatory cells, like macrophages, in DN [24].

Therefore, in the present study, we evaluated the direct or indirect renoprotective effect of sildenafil, focusing on these nitro-oxidative stress and inflammatory markers in the kidneys of STZ-induced diabetic rats.

Materials and Methods

Experimental Animals

Studies were performed in male Sprague-Dawley rats (Central Research Laboratory, Seoul, Korea), weighing 230–290 g. Rats were rendered diabetic by a single intravenous injection of 60 mg/kg/body weight STZ (Sigma, Deisenhofen, Germany) dissolved in 1 ml sodium citrate buffer at 4°C. Only rats with blood glucose concentrations above 250 mg/dl 3 days after induction of diabetes were included in this study. Diabetic (n = 16) and age-matched, nondiabetic (n = 8) rats were followed for 8 weeks.

Diabetic rats were treated with insulin to maintain body weight, prevent ketoacidosis and improve survival. One to four units of ultralente insulin (Ultratard HM; Novo-Nordisk Pharmaceuticals, Bagsvaerd, Denmark) were administered daily to each diabetic rat and blood glucose was monitored weekly (at 8 AM) in all diabetic rats. Diabetic rats were divided into 2 groups: 8 rats with (sildenafil group) or without (nonsildenafil group) sildenafil treatment, each. Sildenafil (Viagra; Pfizer) was administered at a dose of 3 mg/kg/day in drinking water for 8 weeks. In preliminary experiments, this dose was found to be the dose that the rats would tolerate without losing weight or showing deterioration of their general condition. Blood glucose levels were detected weekly and the average glucose levels compared among the 3 groups.

Rats were placed in metabolic cages for 24 h to determine urinary albumin excretion rates by radioimmunoassay at the end of the study. Systolic blood pressure was measured weekly using tail cuff plethysmography. Measurements were performed in a quiet environment in the morning with animals in an unanesthetized preheated state. At the end of the study, after sacrifice, we checked each animal's kidney and body weight for comparison of kidney-to-body weight ratios among the 3 groups. All experiments involving animals were performed according to the guidelines of the animal research ethics committee of Kyung Hee Medical University.

Measurement of Urinary and Renal Cortical 8-OHdG

Urine samples were centrifuged at 2,000 g for 20 min, and after proper dilution, the supernatant was used for the determination of 8-OHdG using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Japan Institute for the Control of Aging, Fukuroi, Japan). Creatinine was also measured in urine samples to correct for muscle mass and dehydration differences between the animals. The kidney was rapidly excised and separated into cortices and papillae. Then, the samples were frozen in liquid nitrogen and kept at -80°C until analyzed. Extraction of renal cortical DNA was performed using a DNA extraction kit (Wako Pure Chemical Industries, Chuoku, Japan) following the manufacturer's protocol. The genomic DNA samples from kidney tissue were also used for the determination of 8-OHdG using the competitive ELISA kit, as described above.

Isolation of Total RNA, Synthesis of cDNA and RT-PCR

Total RNA was extracted from rat kidney cortices with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, Calif., USA). cDNA was synthesized with a reverse transcriptase reaction using standard techniques (SuperScriptTM First-Strand Synthesis System for RT-PCR; Invitrogen Life Technologies) with random hexamers, dNTPs and total RNA extract from control and diabetic rat kidneys. The primer pairs were chosen from the published cDNA sequences of rat iNOS (216 bp) and MCP-1 (929 bp). The primer sequences for iNOS were AAC AGG AAC CTA CCA GCT CA (sense) and AAC ACA GTA ATG GCC GAC CT (antisense); for MCP-1, they were CCG AGA TGT TCC CAG CAC AG (sense) and CTG CTT TGC TTG TGC CTC TT (antisense). For the semi-quantitative analysis of mRNA expression of iNOS, MCP-1 and cyclophilin, the fluorescence intensity of the ethidium bromide-stained RT-PCR products was screened and analyzed using quantitation analysis computer software (Quantity One, Bio-Rad, Hercules, Calif., USA).

Immunohistochemistry

For immunohistochemistry, tissue samples were immediately fixed in 10% buffered formalin and embedded in paraffin. Antigen retrieval was performed with a pressure cooker at 120°C in target retrieval solution, as previously described [25]. Endogenous peroxidase activity was blocked with incubation of the slides in 0.3% H_2O_2 in 100% methanol for 30 min. To block nonspecific binding, sections were incubated at room temperature for 30 min in PBS containing 1% milk and 3% donkey serum. Sections were then incubated for 1 h at room temperature in a humid chamber with the primary iNOS antibody (NOS2 c-11, 1:50 dilution, 0.5% bovine serum albumin in PBS buffer; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), the primary ED-1 antibody (1:100 dilution, 0.5% bovine serum albumin in PBS buffer; Serotec, Oxford, UK) as a marker of monocyte/macrophage infiltration and phagocytic activity and the primary nitrotyrosine antibody (1:100 dilution, 0.5% bovine serum albumin in PBS buffer; Upstate, New York, N.Y., USA) as indirect evidence of peroxynitrite production. After incubation with the primary antibody, the slides were incubated for 60 min at room temperature in a humidified chamber with a secondary antibody. Finally, the slides were incubated with 50 μl diaminobenzadine (BioGenex, San Ramon, Calif., USA) as substrate.

The slides were counterstained with hematoxylin (Sigma), dehydrated, and fixed with Permount histological mounting me-

dium (Fisher Scientific, Pittsburgh, Pa., USA). Immunohistochemical staining intensity for iNOS and nitrotyrosine in glomeruli as well as interstitium was assessed by semiquantitative scoring on a scale of 0–4 as follows: 0 = absent; 1 = occasional; weak; 2 = weak; 3 = moderate; 4 = strong staining. Sections were analyzed by 1 pathologist and 2 nephrologists blinded to the experimental groups, who assessed staining intensity of 100 glomeruli and 20 tubulointerstitial fields in renal cortex from each slide. Quantitative analysis of ED-1-positive cells in glomeruli was performed under a magnification of $\times 400$ and expressed as cells per glomerular cross-section (GCS). For each section, 50 sequential glomerular profiles were examined. ED-1-positive cells in tubulointerstitium were counted in 25 consecutive high-power ($\times 400$) interstitial fields by means of a 0.02-mm² graticule fitted in the eyepiece of the microscope and expressed as cells per square millimeter [26].

Western Blotting Studies

Renal cortical homogenate was used to quantify nitrotyrosine by Western blot analysis. The protein concentration of tissue was determined using a Bio-Rad protein kit and bovine serum albumin as a standard. A total of 25 μg protein was separated by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride membranes (HybondTM-P; Amersham, Piscataway, N.J., USA). The membranes were blocked with 5% (w/v) nonfat dried milk in TBST buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 2 h at room temperature. After blocking of nonspecific binding, the membranes were incubated with a mouse monoclonal anti-nitrotyrosine antibody (1:1,000; Upstate) overnight at 4°C . After washing with TBST buffer, the membranes were incubated with goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Proteins were detected by enhanced chemiluminescence Western blotting substrate (Fisher Scientific) on Hyperfilm (Amersham), according to the manufacturer's instructions. To control for protein loading, all membranes were stripped and probed with a monoclonal anti- β -actin antibody (1:10,000; Sigma) that recognizes the β -actin protein at 43 kDa.

Statistical Analysis

Differences among groups were evaluated by the nonparametric Kruskal-Wallis test and Dunn's post tests. Differences yielding $p < 0.05$ were considered statistically significant. All data are presented as means \pm SE.

Results

Physical and Biochemical Parameters

As shown in table 1, average blood glucose levels during the study period were significantly higher in the diabetic compared with nondiabetic rats (434.3 ± 35.1 vs. 113.0 ± 2.0 mg/dl; $p < 0.05$) and were not significantly affected after sildenafil administration in diabetic rats (433.9 ± 16.7 mg/dl). In the diabetic rats, urine output was markedly higher than that of nondiabetic rats. Mean arterial pressure and serum creatinine levels did not dif-

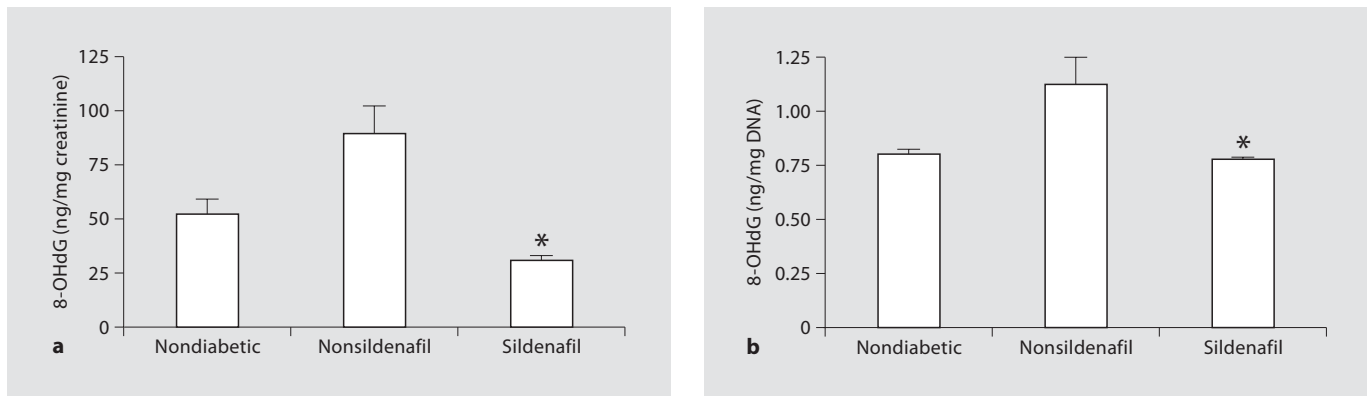


Fig. 1. 8-OHdG levels in urine (a) and renal cortex (b). Urinary and renal tissue 8-OHdG levels in diabetic rats were significantly attenuated after sildenafil administration. * $p < 0.05$ compared with the nonsildenafil group.

Table 1. Physical and biochemical parameters

Group	Blood glucose mg/dl	Kidney-to-body weight ratio, mg/g	Diuresis ml/24 h	Blood pressure mm Hg	Serum creatinine mg/dl	Albuminuria $\mu\text{g}/24\text{ h}$
Nondiabetic	113.0 \pm 2.0	8.0 \pm 0.8	5.3 \pm 1.5	111.3 \pm 3.8	0.54 \pm 0.02	19.9 \pm 8.2
Nonsildenafil	434.3 \pm 35.1 ^a	11.9 \pm 0.1 ^a	14.3 \pm 1.2 ^a	106.3 \pm 1.7	0.56 \pm 0.06	378.0 \pm 146.8 ^a
Sildenafil	433.9 \pm 16.7 ^a	9.8 \pm 0.5 ^b	16.2 \pm 1.8 ^a	112.5 \pm 5.2	0.69 \pm 0.01	79.5 \pm 22.9 ^b

^a $p < 0.05$ compared with the age-matched nondiabetic group. ^b $p < 0.05$ compared with the nonsildenafil group.

fer between diabetic and nondiabetic rats. The kidney-to-body weight ratio of diabetic rats was higher than that of nondiabetic rats (11.9 \pm 0.1 vs. 8.0 \pm 0.8 mg/g; $p < 0.05$), although diabetic rats had lower weight gain at the end of 8 weeks. In diabetic rats, urinary albumin excretion was higher than that of nondiabetic rats (378.0 \pm 146.8 vs. 19.9 \pm 8.2 $\mu\text{g}/24\text{ h}$; $p < 0.05$).

After 8 weeks, treatment with sildenafil was associated with a significant lowering of kidney-to-body weight ratio compared with the non-sildenafil-treated diabetic rats (9.8 \pm 0.5 vs. 11.9 \pm 0.1 mg/g; $p < 0.05$). Sildenafil treatment also reduced albuminuria compared to the nonsildenafil group (79.5 \pm 22.9 vs. 378.0 \pm 146.8 $\mu\text{g}/24\text{ h}$; $p < 0.05$) without a significant reduction in systolic blood pressure.

Urinary 8-OHdG Excretion and 8-OHdG Contents in Renal Cortex

Figure 1 shows the urinary 8-OHdG excretion and 8-OHdG contents in renal cortex. Urinary 8-OHdG levels

were significantly lower in the sildenafil-treated group than in the nonsildenafil group (31.0 \pm 4.0 vs. 89.6 \pm 13.3 ng/mg creatinine; $p < 0.05$). Levels of 8-OHdG in the DNA were also significantly lower in renal cortex of sildenafil-treated diabetic rats compared with those from the nonsildenafil group (0.78 \pm 0.01 vs. 1.12 \pm 0.12 ng/mg DNA; $p < 0.05$).

Immunohistochemical Staining and mRNA Expression for iNOS

Immunohistochemical staining was performed to localize iNOS in the kidney (fig. 2). In the kidneys of nondiabetic animals, iNOS was not expressed at all. It was expressed more intensely in renal tubules and glomeruli of diabetic rats and was significantly reduced after sildenafil administration (glomerulus, sildenafil group: 0.28 \pm 0.18, nonsildenafil group: 2.20 \pm 0.48, nondiabetic group: 0.00 \pm 0.00; tubulointerstitium, sildenafil group: 0.57 \pm 0.20, nonsildenafil group: 3.40 \pm 0.40, nondiabetic group: 0.00 \pm 0.00; $p < 0.05$).

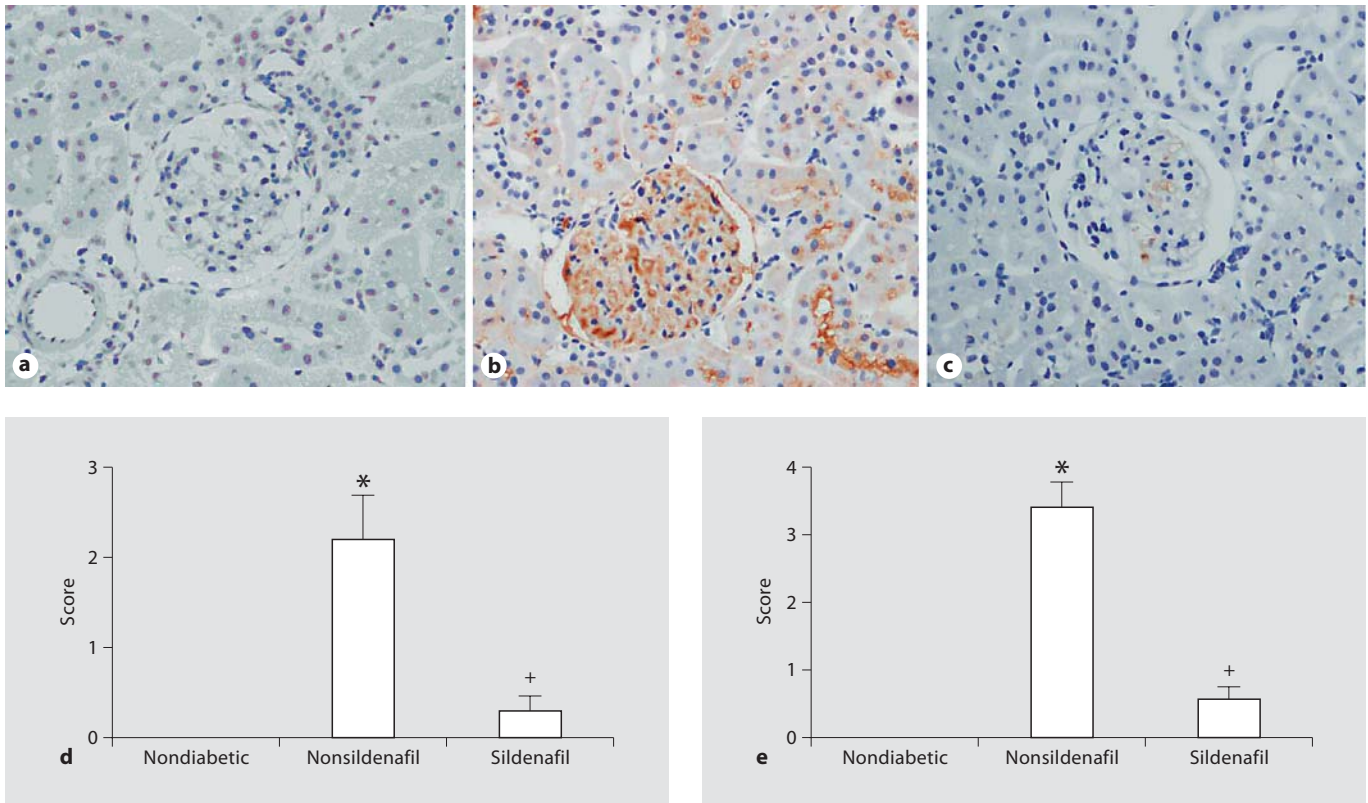


Fig. 2. Immunohistochemistry for iNOS in nondiabetic rats (a), diabetic rats without sildenafil treatment (b) and diabetic rats with sildenafil treatment (c), as well as semiquantitative scoring for glomeruli (d) and the tubulointerstitial area (e). iNOS was expressed more intensely in renal tubulointerstitium and glomeruli of diabetic rats, and this expression was significantly reduced after sildenafil administration. * $p < 0.05$ compared with the age-matched nondiabetic group; + $p < 0.05$ compared with the nonsildenafil group.

There was, however, no significant difference in iNOS mRNA expression among the 3 groups (sildenafil group: 1.04 ± 0.04 , nonsildenafil group: 0.95 ± 0.07 , nondiabetic group: 1.00 ± 0.06 ; $p > 0.05$).

Immunohistochemical Staining and Western Blotting for Nitrotyrosine

There were no significant differences in glomerular nitrotyrosine staining among the 3 groups. Tubulointerstitial staining for nitrotyrosine, however, was less intense in sildenafil-treated diabetic rats than in the nonsildenafil group (1.50 ± 0.22 vs. 3.50 ± 0.22 ; $p < 0.05$; fig. 3).

Figure 4 shows the results of the Western blot analysis for renal nitrotyrosine protein. Renal cortical nitrotyrosine protein was significantly increased in diabetic rats compared with nondiabetic rats (1.00 ± 0.19 vs. 2.87 ± 0.53 ; $p < 0.05$). In addition, the level in sildenafil-treated diabetic rats was significantly less than that

in the nonsildenafil group (1.38 ± 1.02 for the latter; $p < 0.05$).

MCP-1 mRNA Expression

A single transcript of 929 bp was amplified for MCP-1 and 300 bp for cyclophilin (fig. 5). In diabetic rats, cortical MCP-1 expression was higher than in nondiabetic rats (1.63 ± 0.05 vs. 1.00 ± 0.23 ; $p > 0.05$), which was not statistically significant. MCP-1 expression in diabetic rats was, however, significantly attenuated after sildenafil treatment (0.52 ± 0.08 vs. 1.63 ± 0.05 ; $p < 0.05$).

Immunohistochemical Staining for ED-1

ED-1 (CD68) expression levels correlate with phagocytic activity. In the kidney sections of nondiabetic rats, very few macrophages were detected in glomeruli and tubulointerstitium. In diabetic rats, on the other hand, macrophage infiltration was facilitated in glomeruli and

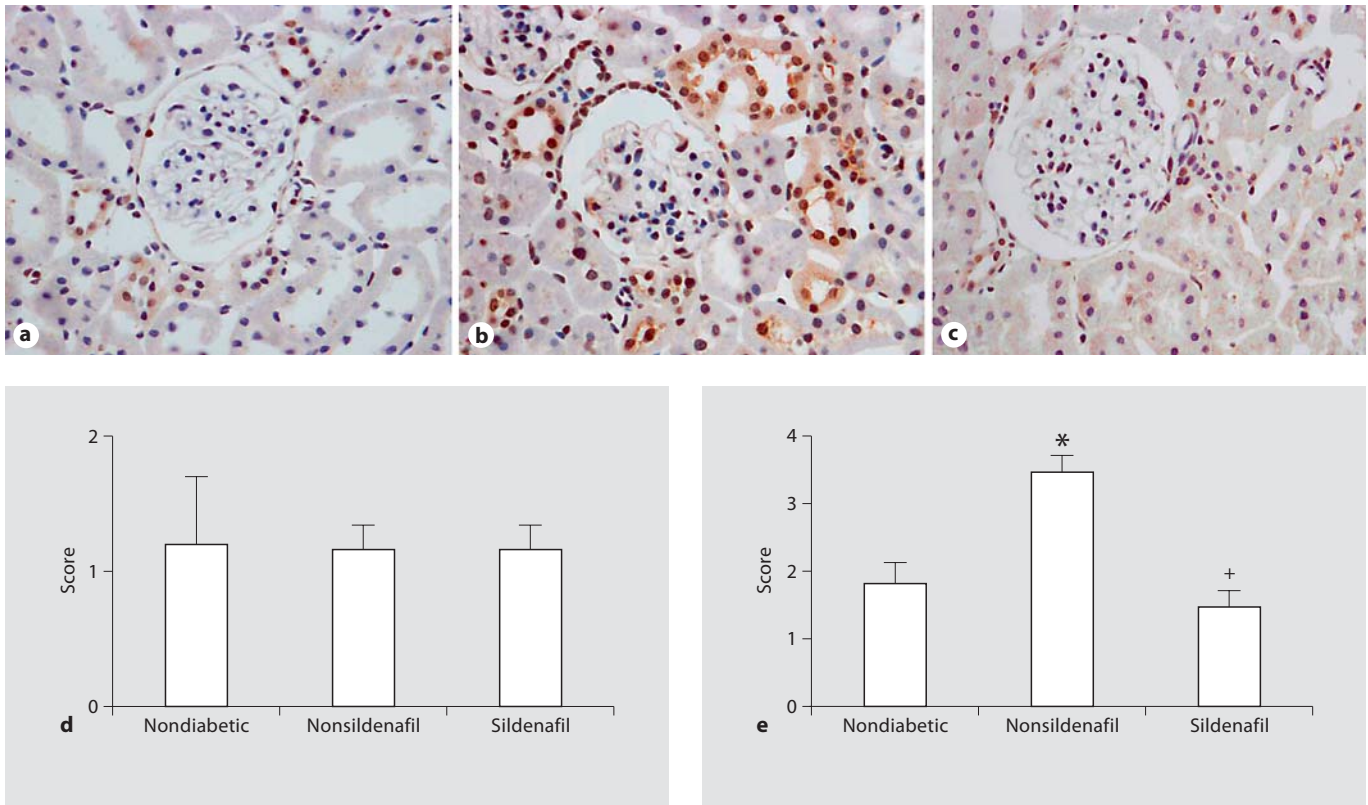


Fig. 3. Immunohistochemistry for nitrotyrosine in nondiabetic rats (a), diabetic rats without sildenafil treatment (b) and diabetic rats with sildenafil treatment (c), as well as semiquantitative scoring for glomeruli (d) and the tubulointerstitial area (e). In sildenafil-treated diabetic rats, tubulointerstitial staining for nitrotyrosine was less intense than in untreated diabetic animals. * $p < 0.05$ compared with age-matched nondiabetic animals; + $p < 0.05$ compared with the nonsildenafil group.

tubulointerstitium. Treatment with sildenafil ameliorated the STZ-induced macrophage infiltration in both areas (glomerulus, sildenafil group: $0.83 \pm 0.30/\text{GCS}$, nonsildenafil group: $2.50 \pm 0.22/\text{GCS}$, nondiabetic group: $0.33 \pm 0.21/\text{GCS}$; tubule, sildenafil group: $0.83 \pm 0.30/\text{mm}^2$, nonsildenafil group: $2.33 \pm 0.21/\text{mm}^2$, nondiabetic group: $0.66 \pm 0.21/\text{mm}^2$; $p < 0.05$; fig. 6).

Discussion

The central finding of the present study is that treatment with sildenafil may retard the progression of nephropathy in the STZ-induced diabetic rat model. Daily administration of sildenafil begun immediately after STZ injection resulted in stabilization of oxidative stress levels, prevention of inflammation and reduction of albuminuria in the STZ-induced animals. It has previously

been reported that administration of sildenafil for 30 consecutive days diminishes microalbuminuria and the percentage of A1c in patients with type 2 diabetes [27]. However, to our knowledge, the current study is the first to demonstrate amelioration of both oxidative stress and inflammatory markers in the kidneys of STZ-induced diabetic rats that were administered sildenafil.

iNOS can produce large amounts of NO that, under oxidative stress conditions, can react with superoxide anion to form peroxynitrite, an oxidant species able to modify a great number of biomolecules, such as amino acids, proteins and cofactors [28]. Interestingly, peroxynitrite can modify tyrosine residues in various proteins to form nitrotyrosine, and nitration of protein tyrosine residues can lead to damage that alters protein function and stability [29]. Therefore, we speculate that sildenafil may decrease the generation of peroxynitrite by downregulating iNOS expression in STZ-induced diabetic rat kidneys.

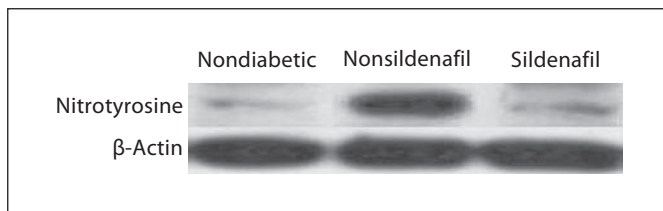
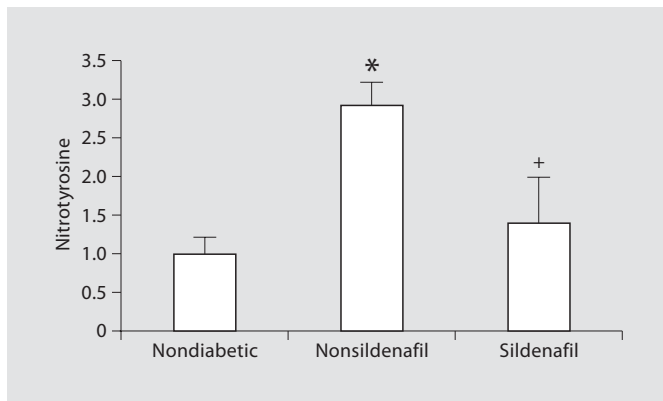


Fig. 4. Western blot analysis of renal cortical nitrotyrosine. Representative Western blots and densitometric analysis. * $p < 0.05$ compared with the age-matched nondiabetic group; + $p < 0.05$ compared with the nonsildenafil group.

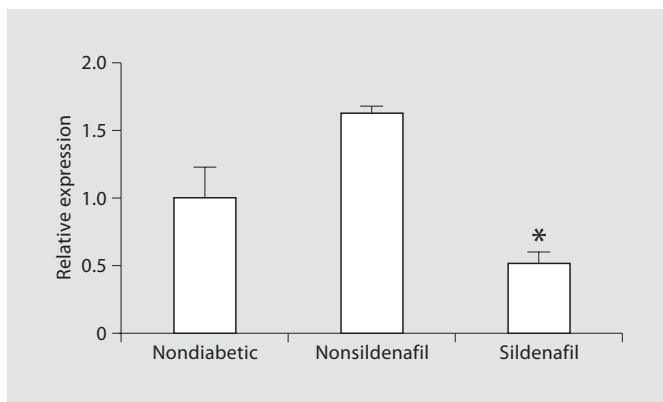


Fig. 5. Quantification of MCP-1 mRNA expression from renal cortex. In diabetic rats, cortical MCP-1 expression was significantly attenuated after sildenafil treatment. * $p < 0.05$ compared with the nonsildenafil group.

However, RT-PCR revealed no obvious differences in iNOS mRNA expression among the groups in this study, and the etiology of these differences remains unknown. One possibility is that the discrepancy between immunohistochemical staining in tissue and mRNA expression

quantification probably indicates a posttranscriptional dysregulation. These hypotheses require further evaluation.

The production of 8-OHdG as a measure of DNA oxidation has led to a novel way of detecting the oxidative DNA damage that is often observed in diabetes [30]. Administration of sildenafil effectively attenuated the diabetes-induced increase in renal 8-OHdG levels in this study. This result suggests that sildenafil could inhibit the development of diabetic nephropathy, in part, via inhibiting accumulation of oxidized DNA in the kidney.

Although the pathogenetic mechanism of DN has not been fully elucidated, an inflammatory mechanism has been suggested to contribute to its progression. Macrophages can be activated directly by mechanical stress and hyperglycemia and may produce inflammatory mediators, thus recruiting additional inflammatory cells to contribute to the propagation of glomerular and tubulointerstitial injuries [31, 32]. Ihm et al. [24] demonstrated that high glucose can directly increase MCP-1 expression in mesangial cells, which may contribute to monocyte infiltration in DN. In the present study, increased macrophage infiltration in glomeruli and tubulointerstitium shown by ED-1-positive cells was found to correlate with MCP-1 expression. Sildenafil treatment suppressed the infiltration of ED-1-positive cells into the glomeruli and tubulointerstitium and inhibited increased MCP-1 expression in the diabetic cortex. The exact mechanism by which sildenafil attenuates upregulation of MCP-1 expression in diabetes remains to be determined.

Several potential explanations may be offered for the beneficial effects of sildenafil on DN in this study. The administration of sildenafil has been shown to attenuate tubulointerstitial inflammation in 5/6 nephrectomy animals [15], a finding that parallels the results of the present study. In addition, as shown by Muzaffar et al. [16], a PDE-5 inhibitor reduces superoxide formation and gp91phox expression in vitro, and it is possible that sildenafil could ameliorate oxidative stress in the diabetic kidney. These renoprotective effects on inflammatory stimuli and against oxidative stress indicate that sildenafil is likely to be acting, in part, at one or more central and convergent points of inflammatory and oxidative signaling. However, the mechanisms causing these effects of sildenafil remain unclear. Previous work suggested that a pathologic hypoxic condition occurs in the diabetic renal medulla and is associated with ROS formation [33]. Possibly, if the increase in the pool of cGMP by a PDE-5 inhibitor modulates the vascular tone and improves hypox-

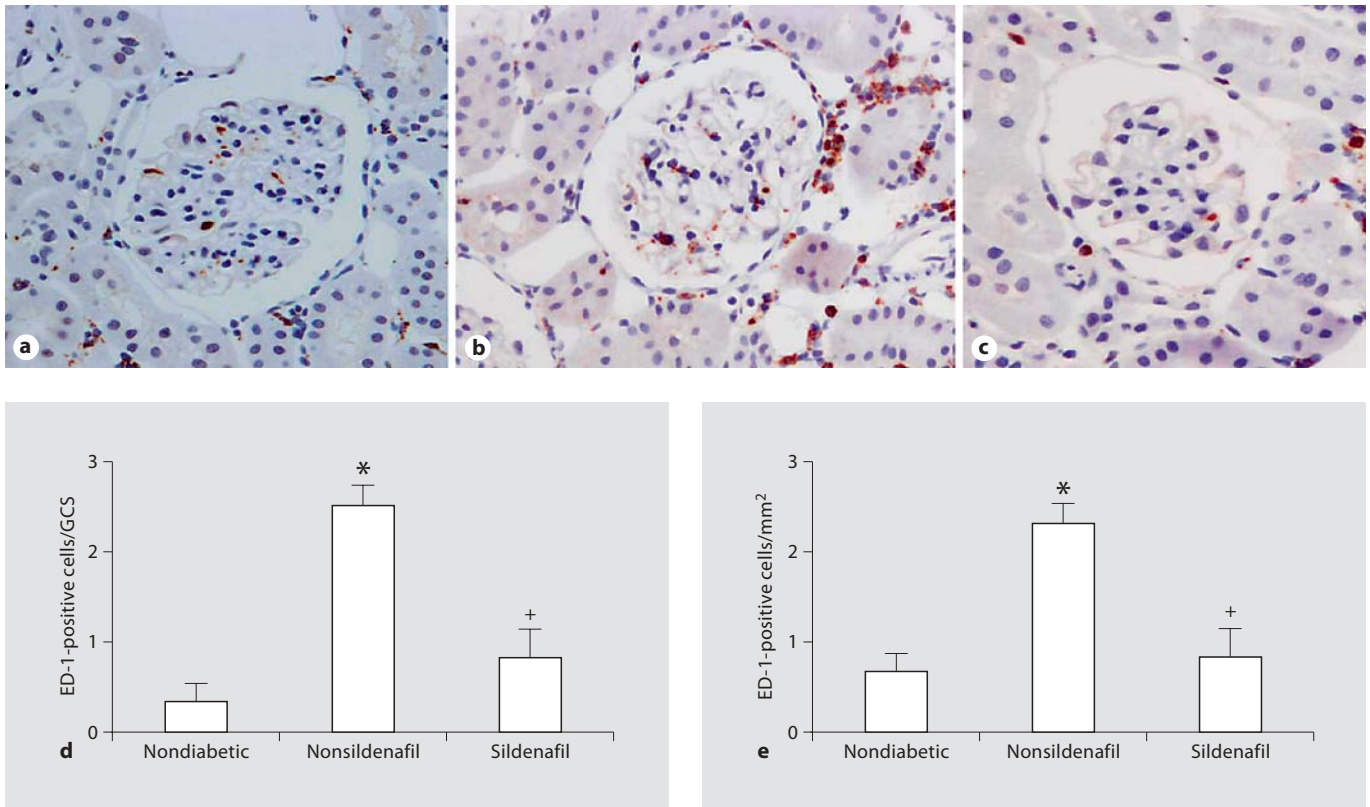


Fig. 6. Immunohistochemistry for ED-1 in nondiabetic rats (a), diabetic rats without sildenafil treatment (b) and diabetic rats with sildenafil treatment (c), as well as scoring for glomeruli (d) and the tubulointerstitial area (e). In diabetic rats, macrophage infiltration was facilitated in glomeruli and tubulointerstitium. Treatment with sildenafil ameliorated the STZ-induced macrophage infiltration in both areas. * $p < 0.05$ compared with the age-matched nondiabetic group; + $p < 0.05$ compared with the nonsildenafil group.

ia, it may contribute to the observed attenuation of oxidative stress and inflammation in the sildenafil-treated DN animals.

In conclusion, sildenafil prevents renal injury by the attenuation of the oxidative and inflammatory mecha-

nisms of renal damage in DN. Further research is needed to delineate the exact mechanisms involved in sildenafil-mediated prevention of DN to allow for translation into the clinical setting.

References

- 1 Son SM, Whalin MK, Harrison DG, Taylor WR, Griendling KK: Oxidative stress and diabetic vascular complications. *Curr Diab Rep* 2004;4:247-252.
- 2 Schnackenberg CG: Physiological and pathophysiological roles of oxygen radicals in the renal microvasculature. *Am J Physiol Regul Integr Comp Physiol* 2002;282:R335-R342.
- 3 Lee EA, Seo JY, Jiang Z, Yu MR, Kwon MK, Ha H, Lee HB: Reactive oxygen species mediate high glucose-induced plasminogen activator inhibitor-1 up-regulation in mesangial cells and in diabetic kidney. *Kidney Int* 2005;67:1762-1771.
- 4 Baldwin AS Jr: The NF- κ B and I κ B proteins: new discoveries and insights. *Annu Rev Immunol* 1996;14:649-683.
- 5 Chow FY, Nikolic-Paterson DJ, Atkins RC, Tesch GH: Macrophages in streptozotocin-induced diabetic nephropathy: potential role in renal fibrosis. *Nephrol Dial Transplant* 2004;19:2987-2996.
- 6 Van Goor H, Ding G, Kees-Folts D, Grond J, Schreiner GF, Diamond JR: Macrophages and renal disease. *Lab Invest* 1994;71:456-464.

- 7 Forbes JM, Fukami K, Cooper ME: Diabetic nephropathy: where hemodynamics meets metabolism. *Exp Clin Endocrinol Diabetes* 2007;115:69–84.
- 8 Modlinger PS, Wilcox CS, Aslam S: Nitric oxide, oxidative stress, and progression of chronic renal failure. *Semin Nephrol* 2004; 24:354–365.
- 9 Jeremy JY, Ballard SA, Naylor AM, Miller MA, Angelini GD: Effects of sildenafil, a type-5 cGMP phosphodiesterase inhibitor, and papaverine on cyclic GMP and cyclic AMP levels in the rabbit corpus cavernosum in vitro. *Br J Urol* 1997;79:958–963.
- 10 Rosen RC, Kostis JB: Overview of phosphodiesterase 5 inhibition in erectile dysfunction. *Am J Cardiol* 2003;92:9M–18M.
- 11 Lin CS, Lin G, Xin ZC, Lue TF: Expression, distribution and regulation of phosphodiesterase 5. *Curr Pharm Des* 2006;12:3439–3457.
- 12 Wallis RM, Corbin JD, Francis SH, Ellis P: Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. *Am J Cardiol* 1999; 83:3C–12C.
- 13 Dousa TP: Cyclic-3',5'-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney Int* 1999;55:29–62.
- 14 Lledo-García E, Rodríguez-Martínez D, Cabello-Benavente R, Moncada-Iribarren I, Tejedor-Jorge A, Dulin E, Hernández-Fernández C, Del Canizo-López JF: Sildenafil improves immediate posttransplant parameters in warm-ischemic kidney transplants: experimental study. *Transplant Proc* 2007; 39:1354–1356.
- 15 Rodríguez-Iturbe B, Ferrebuz A, Vanegas V, Quiroz Y, Espinoza F, Pons H, Vaziri ND: Early treatment with cGMP phosphodiesterase inhibitor ameliorates progression of renal damage. *Kidney Int* 2005;68:2131–2142.
- 16 Muzaffar S, Shukla N, Srivastava A, Angelini GD, Jeremy JY: Sildenafil citrate and sildenafil nitrate (NCX 911) are potent inhibitors of superoxide formation and gp91phox expression in porcine pulmonary artery endothelial cells. *Br J Pharmacol* 2005;146:109–117.
- 17 Cooke MS, Evans MD, Herbert KE, Lunec J: Urinary 8-oxo-2'-deoxyguanosine – source, significance and supplements. *Free Radic Res* 2000;32:381–397.
- 18 Liaudet L, Soriano FG, Szabo C: Biology of nitric oxide signaling. *Crit Care Med* 2000; 28:N37–N52.
- 19 Prabhakar S, Starnes J, Shi S, Lonis B, Tran R: Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production. *J Am Soc Nephrol* 2007; 18:2945–2952.
- 20 Pacher P, Obrosova IG, Mabley JG, Szabo C: Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications: Emerging new therapeutical strategies. *Curr Med Chem* 2005;12:267–275.
- 21 Komers R, Anderson S: Paradoxes of nitric oxide in the diabetic kidney. *Am J Physiol Renal Physiol* 2003;284:F1121–F1137.
- 22 Trachtman H, Futterweit S, Pine E, Mann J, Valderrama E: Chronic diabetic nephropathy: role of inducible nitric oxide synthase. *Pediatr Nephrol* 2002;17:20–29.
- 23 Boyle JJ: Macrophage activation in atherosclerosis: Pathogenesis and pharmacology of plaque rupture. *Curr Vasc Pharmacol* 2005; 3:63–68.
- 24 Ihm CG, Park JK, Hong SP, Lee TW, Cho BS, Kim MJ, Cha DR, Ha H: A high glucose concentration stimulates the expression of monocyte chemotactic peptide 1 in human mesangial cells. *Nephron* 1998;79:33–37.
- 25 Brown C: Antigen retrieval methods for immunohistochemistry. *Toxicol Pathol* 1998; 26:830–831.
- 26 Mai M, Geiger H, Hilgers KF, Veelken R, Mann JF, Dammrich J, Luft FC: Early interstitial changes in hypertension-induced renal injury. *Hypertension* 1993;22:754–765.
- 27 Grover-Paez F, Villegas Rivera G, Guillen Ortiz R: Sildenafil citrate diminishes microalbuminuria and the percentage of A1c in male patients with type 2 diabetes. *Diabetes Res Clin Pract* 2007;78:136–140.
- 28 Szabo C: Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett* 2003;140–141:105–112.
- 29 Gow AJ, Duran D, Malcolm S, Ischiropoulos H: Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett* 1996;385:63–66.
- 30 Wu LL, Chiou CC, Chang PY, Wu JT: Urinary 8-OHdG: A marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. *Clin Chim Acta* 2004;339:1–9.
- 31 Mensah-Brown EP, Obineche EN, Galadari S, Chandranath E, Shahin A, Ahmed I, Patel SM, Adem A: Streptozotocin-induced diabetic nephropathy in rats: the role of inflammatory cytokines. *Cytokine* 2005;31:180–190.
- 32 Galkina E, Ley K: Leukocyte recruitment and vascular injury in diabetic nephropathy. *J Am Soc Nephrol* 2006;17:368–377.
- 33 Rosenberger C, Khamaisi M, Abassi Z, Shilo V, Weksler-Zangen S, Goldfarb M, Shina A, Zibertrest F, Eckardt KU, Rosen S, Heyman SN: Adaptation to hypoxia in the diabetic rat kidney. *Kidney Int* 2008;73:34–42.